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Prebiotic potential of neutral oligo- and polysaccharides from seed mucilage of *Hyptis suaveolens*

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ABSTRACT

Prebiotics are selectively fermented by the gastrointestinal microflora, resulting in benefits to human health. The seed mucilage of *Hyptis suaveolens* contains neutral and acidic polysaccharides in a ratio of 1:1. The neutral polysaccharides consist of galactose, glucose and mannose whereas the acidic polysaccharides contain fucose, xylose and 4-*O*-methylglucuronic acid -residues. The growth of probiotics in the presence of total, acidic or neutral polysaccharides and oligosaccharides was tested using turbidity measurements. The majority (11 out of 14) of the tested probiotic strains significantly grew in the neutral fraction. Growth occurred with some time delay, but may be longer lasting than with other lower molecular prebiotics. The extent of growth increased with neutral polysaccharides from *H. suaveolens* corresponding to the externally available galactose units (20%). In conclusion, neutral poly- and oligosaccharides from *H. suaveolens* have a prebiotic potential characterized by a delayed but long lasting effect.

1. Introduction

Hyptis suaveolens (Lamiaceae), commonly named bush mint, pignut, chan, sangura and wilaiti tulsi is an annual aromatic herb widespread in Latin America, India, China, Australia, Central and South Africa where it is commonly used in food or as a source of essential oil (Aguirre, Torres, Mendoza-Hernandez, Garcia-Gasca, & Blanco-Labra, 2012; Ngozi, Ugochukwu, Ifeoma, Charity, & Chinyelu, 2014; Purnima, 2006; Sharma Prince, Roy Ram, Anurag, Gupta, & Vipin, 2013). Furthermore, it is traditionally used for the treatment of respiratory illness, skin diseases, and gastrointestinal disorders in Asia, Africa and South America (Aguirre et al., 2012; Jesus et al., 2013; Ngozi et al., 2014; Sharma Prince et al., 2013). The essential oil is a potent mosquito repellent (Abagli, Alavo, Avlessi, & Moudachirou, 2012). The seeds contain proteins with a high content of aromatic and branched-chain amino acids and are a good source of mineral nutrients (Aguirre et al., 2012). The fatty acid profile shows the presence of saturated fatty acids and a high content of polyunsaturated linoleic acid (Ngozi et al., 2014). In addition, the polysaccharides, which have great water binding capacity, and can be extracted in approximately 12% yield from the normally very viscous, almost tasteless and odorless seed mucilage, show great potential as binding, gelling, stabilizing and thickening agents. These properties raise the question of whether mucilages from *H. suaveolens* may function as prebiotics.

A dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, which benefits the host's well-being and health (Roberfroid et al., 2010). Prebiotics are stable under acidic gastric conditions, escape digestion in the upper gastrointestinal tract and alter the bacterial composition of the gut by changing the type of substrate provided to the existing gut microbiota (Gibson & Roberfroid, 1995; Roberfroid et al., 2010). So far, only a few prebiotics fully meet this definition including fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS), and gluco-oligosaccharides (Roberfroid et al., 2010).

Several nondigestible food ingredients are emerging candidates for prebiotics including lactulose, galactans, fructans, xylo-oligosaccharides, β-glucans or arabinoxylans (Cummings, Macfarlane, & Englyst, 2001; Mei, Carey, Tosh, & Kostrzynska, 2011). The prebiotic properties of carbohydrates are influenced by monosaccharide composition, the glycosidic linkages between the monosaccharide residues and the degree of polymerization (DP) (Manning & Gibson, 2004; Mueller, Reiner, Viernstein, Loeppert, & Praznik, 2016; Mueller, Schwarz, Viernstein, Loeppert, & Praznik, 2016).

Seed mucilage of *H. suaveolens* contains neutral and acidic polysaccharides in the ratio of 1:1. The monosaccharide

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composition of the neutral polysaccharide is galactose, glucose and mannose, whereas the highly branched acidic polysaccharide is composed of fucose, xylose and 4-*O*-methylglucuronic acid as described previously (Aspinall, Capek, Carpenter, Gowda, & Szafranek, 1991; Gowda, 1984; Praznik, Cavarcapa, Loeppert, Unger, Viernstein, & Mueller, 2016).

The aim of this study was to determine the prebiotic potential of poly- and oligosaccharides isolated from the seed mucilage of *H. suaveolens* by investigating the impact of separation into acidic and neutral fractions and the effect of enzymatic treatment.

2. Materials and methods

2.1. Materials

The seeds of *Hyptis suaveolens* L. were collected from plants cultivated in the north of Thailand. The mucilage of seeds was prepared in cooperation with the Chiang Mai University of Thailand. The mucilage was extracted using cold water, then purified and dried at low temperature, in preparation for application as the basic material for the investigations. Potato galactan and galactanase were obtained from Megazyme (Wicklow, Ireland). Yeast extract was obtained from Oxoid (Hampshire, UK). All chemicals for the extraction, separation and purification of the polysaccharides or for the Man-Rogosa-Sharp (MRS) medium were purchased from Sigma-Aldrich (St. Louis, MA, USA). MRS was prepared as described previously (Mueller, Reiner et al., 2016). The probiotic strains used in this study included *Lactobacillus* (*L.*) *paracasei ssp. paracasei* CRL 431 (ATCC 55544), *L. paracasei ssp. paracasei* DN114001, *L. paracasei ssp. paracasei* DSM 20312, *L. rhamnosus* GG (ATCC 53103), *L. reuteri* (ATCC 55730), *L. acidophilus* LA-5 (DSM 13241), *B. animalis ssp. lactis BB12* (DSM 15954), *B. longum ssp. infantis*, *L. plantarum* (ATCC 15697), *L. brevis* (ATCC 367), *L. bulgaricus* (DSM 20081), *Lactococcus* (*Lc*) *lactis ssp. lactis* (SR 3.54; NCIMB 30117), *L. fermentum* and *Streptococcus* (*S.*) *thermophilus* (ATCC 19258).

2.2. Hydrolysis of potato galactan

Potato galactan is highly polymeric and thus required hydrolysis to be fermentable for probiotics. This was performed using galactanase in aqueous solution at 38 °C for 24 h. The hydrolysate was used to test the β-galactosidase activity in the bacteria.

2.3. Extraction of the alkali-soluble polysaccharides and separation into neutral and acidic fractions

The total soluble polysaccharides (TPS), the fraction of neutral polysaccharides (NPS) and the fraction of acidic polysaccharides (APS) were obtained as described in the first part of these investigations (Praznik et al., 2016). In brief, finely milled mucilage was treated with 4 M sodium hydroxide solution (NaOH) for 24 h at 70 °C. After dilution with distilled water and centrifugation to remove the insoluble parts, the precipitate was washed twice with 2 M NaOH solution. After neutralization, the clear solution was dialyzed against distilled water using a cellulose membrane with a cut off of 13.4 kDa and finally lyophilized to obtain TPS.

Alkali-soluble polysaccharides, dissolved in 0.005 M formate buffer, were applied to an anion exchange column (Toyopearl TSK DEAE-650(M), 1.5 × 20 cm, flow rate 1 ml.min^{-1}). The neutral polysaccharides (NPS) were eluted with this buffer; the respective fractions were pooled and lyophilized. The fraction of acidic polysaccharides (APS) was subsequently eluted in 0.35 M formate buffer.

2.4. Enzymatic treatment and fractionation of NPS

The enzymatic treatment and fractionation was performed as described in the first part of the investigations (Praznik et al., 2016). In brief, NPS dissolved in bi-distilled water was incubated for 48 h with either the purified exo-β-galactosidase (from *Aspergillus niger*, Megazyme) (10 U/ml each) at 38 °C, or with endo-β-1,4-mannanase (from *A. niger*, Megazyme) at 40 °C. The reactions were stopped by heating for 15 min at 80 °C and the inactivated enzymes were removed by centrifugation.

The solutions of the cleaved NPS were separated by preparative size exclusion chromatography (SEC) with a combined column system of Sephacryl S 200 (range 1–400 kDa, 15 × 1000 mm) and Biogel P2 (range 100–2000 Da; 15 × 1200 mm) with bi-distilled water as eluent and a flow rate of 0.7 ml.min^{-1} using HPLC pump K500 (Knauer, Berlin, Germany) with a Rheodyne injection system. Differential refractive index (DRI) detection (Shodex RI-101, Thermo Fisher, Waltham, MA, USA) with online data acquisition was performed. The collected fractions with respective ranges of molar mass were pooled and lyophilized. Additionally, for combined Biogel P4/P2 columns (800–4000 Da, 25 × 890 mm/100–2000 Da, 25 × 2000 mm) were used for oligosaccharide preparations with bi-distilled water as eluent, at a flow rate of 0.5 ml.min^{-1} with DRI detection. The pooled fractions were lyophilized and used to determine the prebiotic effect, as described later.

2.5. Determination of the molecular weight by analytical SEC

Analytical methods are described in part 1 of the investigations Praznik et al. (2016). In brief, for analytical SEC 300 µl solutions of the polysaccharide or fractions (10 mg of each in 1 ml 0.05 M sodium chloride) were injected into a system of four combined columns: Toyopearl HW 40S (pre column, 10 × 150 mm), Superose 6, Superose 12, and Toyopearl HW 40S (10 × 300 mm of each); flow rate of 0.6 ml/min and a pressure of 2 bars using HPLC pump K500 (Knauer,), Rheodyne injection system and DRI detection (Shodex RI-101). The system was calibrated with dextrans (Pharmacosmos dextran standard kit; Dk) of different molar mass in the peak$_m$ (401,300, 123,600, 21,400, 9890 and 1080 Da) and glucose. The molar mass distribution was detected by a DRI detector and computed with the acquisition software CODAWIN. The following moments were computed: weight average molar mass (Mw), number average molar mass (Mn) and polydispersity (PD), and the corresponding weight average and number average degree of polymerization (dp$_w$, dp$_n$).

2.6. Determination of the prebiotic effect

Growth curves of the probiotic strains in the presence of standard compounds and different fractions from *H. suaveolens* were derived using Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland). With this method, the change of the OD_{600} value was measured indicating turbidity and thus bacterial growth. A fresh overnight culture was prepared. The cell amount required for a starting OD_{600} of 0.1 was harvested, washed three times in phosphate buffered saline (PBS) supplemented with 0.5 g/l cystein hydrochloride, and resuspended in MRS-medium without sugar. The negative control was incubated without sugar, whereas the samples were incubated in the presence of 1% sugars, in honeycomb plates at 37 °C for 48 h and the OD_{600} was measured every hour after mixing for 15 s. The growth curves were determined 3 times and the results are shown as a mean. The time after which the medium value of the maximal OD_{600} increase (medium effective time, ET_{50}) was reached, was determined using TableCurve2D software (Systat Software Inc., San Jose, CA, USA).